

**TOTIPOTENT HEMATOPOIETIC STEM CELL  
RECEPTORS AND THEIR LIGANDS**

This application is a continuation-in-part of unassigned serial number filed November 12, 1992, which is a continuation-in-part of serial number 906,397 filed June 26, 1992, which is a continuation-in-part of serial number 813,593 filed December 24, 1991, which is a continuation-in-part of serial number 793,065 filed November 15, 1991, which is a continuation-in-part of serial number 728,913 filed June 28, 1991, which is a continuation-in-part of serial number 679,666 filed April 2, 1991, all of which are incorporated herein by reference.

The invention described in this application was made with U.S. government support from Grant Numbers R01-CA45339 and R01-DK42989 awarded by the National Institutes of Health. The government has certain rights in this invention.

FIELD OF THE INVENTION

The present invention relates to hematopoietic stem cell receptors, ligands for such receptors, and nucleic acid molecules encoding such receptors and ligands.

BACKGROUND OF THE INVENTION

The mammalian hematopoietic system comprises red and white blood cells. These cells are the mature cells that result from more primitive lineage-restricted cells. The cells of the hematopoietic system have been reviewed by Dexter and Spooner in the Annual Review of Cell Biology 3, 423-441 (1987).

The red blood cells, or erythrocytes, result from primitive cells referred to by Dexter and Spooner as erythroid burst-forming units (BFU-E). The immediate progeny of the erythroid burst-forming units are called erythroid colony-forming units

(CFU-E).

The white blood cells contain the mature cells of the lymphoid and myeloid systems. The lymphoid cells include B lymphocytes and T lymphocytes. The B and T lymphocytes result from earlier progenitor cells referred to by Dexter and Spooncer as preT and preB cells.

The myeloid system comprises a number of cells including granulocytes, platelets, monocytes, macrophages, and megakaryocytes. The granulocytes are further divided into neutrophils, eosinophils, basophils and mast cells.

Each of the mature hematopoietic cells are specialized for specific functions. For example, erythrocytes are responsible for oxygen and carbon dioxide transport. T and B lymphocytes are responsible for cell-and antibody-mediated immune responses, respectively. Platelets are involved in blood clotting. Granulocytes and macrophages act generally as scavengers and accessory cells in the immune response against invading organisms and their by-products.

At the center of the hematopoietic system lie one or more totipotent hematopoietic stem cells, which undergo a series of differentiation steps leading to increasingly lineage-restricted progenitor cells. The more mature progenitor cells are restricted to producing one or two lineages. Some examples of lineage-restricted progenitor cells mentioned by Dexter and Spooncer include granulocyte/macrophage colony-forming cells (GM-CFC), megakaryocyte colony-forming cells (Meg-CFC), eosinophil colony-forming cells (Eos-CFC), and basophil colony-forming cells (Bas-CFC). Other examples of progenitor cells are discussed above.

The hematopoietic system functions by means of a precisely

controlled production of the various mature lineages. The totipotent stem cell possesses the ability both to self renew and to differentiate into committed progenitors for all hematopoietic lineages. These most primitive of hematopoietic cells are both necessary and sufficient for the complete and permanent hematopoietic reconstitution of a radiation-ablated hematopoietic system in mammals. The ability of stem cells to reconstitute the entire hematopoietic system is the basis of bone marrow transplant therapy.

It is known that growth factors play an important role in the development and operation of the mammalian hematopoietic system. The role of growth factors is complex, however, and not well understood at the present time. One reason for the uncertainty is that much of what is known about hematopoietic growth factors results from in vitro experiments. Such experiments do not necessarily reflect in vivo realities.

In addition, in vitro hematopoiesis can be established in the absence of added growth factors, provided that marrow stromal cells are added to the medium. The relationship between stromal cells and hematopoietic growth factors in vivo is not understood. Nevertheless, hematopoietic growth factors have been shown to be highly active in vivo.

From what is known about them, hematopoietic growth factors appear to exhibit a spectrum of activities. At one end of the spectrum are growth factors such as erythropoietin, which is believed to promote proliferation only of mature erythroid progenitor cells. In the middle of the spectrum are growth factors such as IL-3, which is believed to facilitate the growth and development of early stem cells as well as of numerous progenitor cells. Some examples of progenitor cells induced by IL-3 include those restricted to the granulocyte/macrophage, eosinophil, megakaryocyte, erythroid and mast cell lineages.

At the other end of the spectrum is the hematopoietic growth factor that, along with the corresponding receptor, was discussed in a series of articles in the October 5, 1990 edition of Cell. The receptor is the product of the W locus, c-kit, which is a member of the class of receptor protein tyrosine kinases. The ligand for c-kit, which is referred to by various names such as stem cell factor (SCF) and mast cell growth factor (MGF), is believed to be essential for the development of early hematopoietic stem cells and cells restricted to the erythroid and mast cell lineages in mice; see, for example, Copeland et al., Cell 63, 175-183 (1990).

It appears, therefore, that there are growth factors that exclusively affect mature cells. There also appear to be growth factors that affect both mature cells and stem cells. The growth factors that affect both types of cells may affect a small number or a large number of mature cells.

There further appears to be an inverse relationship between the ability of a growth factor to affect mature cells and the ability of the growth factor to affect stem cells. For example, the c-kit ligand, which stimulates a small number of mature cells, is believed to be more important in the renewal and development of stem cells than is IL-3, which is reported to stimulate proliferation of many mature cells (see above).

Prior to the present specification, there have been no reports of growth factors that exclusively stimulate stem cells in the absence of an effect on mature cells. The discovery of such growth factors would be of particular significance.

As mentioned above, c-kit is a protein tyrosine kinase (pTK). It is becoming increasingly apparent that the protein tyrosine kinases play an important role as cellular receptors for hematopoietic growth factors. Other receptor pTKs include the

receptors of colony stimulating factor 1 (CSF-1) and PDGF.

The pTK family can be recognized by the presence of several conserved amino acid regions in the catalytic domain. These conserved regions are summarized by Hanks et al. in Science 241, 42-52 (1988), see Figure 1 starting on page 46 and by Wilks in Proc. Natl. Acad. Sci. USA 86, 1603-1607 (1989), see Figure 2 on page 1605.

Additional protein tyrosine kinases that represent hematopoietic growth factor receptors are needed in order more effectively to stimulate the self-renewal of the totipotent hematopoietic stem cell and to stimulate the development of all cells of the hematopoietic system both in vitro and in vivo. Novel hematopoietic growth factor receptors that are present only on primitive stem cells, but are not present on mature progenitor cells, are particularly desired. Ligands for the novel receptors are also desirable to act as hematopoietic growth factors. Nucleic acid sequences encoding the receptors and ligands are needed to produce recombinant receptors and ligands.

#### SUMMARY OF THE INVENTION

These and other objectives as will be apparent to those with ordinary skill in the art have been met by providing isolated mammalian nucleic acid molecules encoding receptor protein tyrosine kinases expressed in primitive hematopoietic cells and not expressed in mature hematopoietic cells. Also included are the receptors encoded by such nucleic acid molecules; the nucleic acid molecules encoding receptor protein tyrosine kinases having the sequences shown in Figure 1a (murine flk-2), Figure 1b (human flk-2) and Figure 2 (murine flk-1); the receptor protein tyrosine kinases having the amino acid sequences shown in Figure 1a, Figure 1b and Figure 2; ligands for the receptors; nucleic acid sequences that encode the ligands; and methods of stimulating the

proliferation of primitive mammalian hematopoietic stem cells comprising contacting the stem cells with a ligand that binds to a receptor protein tyrosine kinase expressed in primitive mammalian hematopoietic cells and not expressed in mature hematopoietic cells.

#### DESCRIPTION OF THE FIGURES

Figure 1a.1 through 1a.6 shows the cDNA and amino acid sequences of murine flk-2. All subsequent references to Figure 1a are intended to refer to Figure 1a.1 through 1a.6. The amino acid residues occur directly below the nucleotides in the open reading frame. Amino acids -27 to -1 constitute the hydrophobic leader sequence. Amino acids 1 to 517 constitute the extracellular receptor domain. Amino acids 518 to 537 constitute the transmembrane region. Amino acids 538 to 966 constitute the intracellular catalytic domain. Counting amino acid residue -27 as residue number 1, the following amino acid residues in the intracellular domain are catalytic sub-domains identified by Hanks (see above): 618-623, 811-819, 832-834, 857-862, 872-878. The sequence at residues 709-785 is a signature sequence characteristic of flk-2. The protein tyrosine kinases generally have a signature sequence in this region. (See SEQ. ID. NOS. 1-2)

Figure 1b.1 through 1b.6 shows the complete cDNA and amino acid sequences of human flk-2 receptor. All subsequent references to Figure 1b are intended to refer to Figure 1b.1 through 1b.6. Amino acids -27 to -1 constitute the hydrophobic leader sequence. Amino acids 1 to 516 constitute the extracellular receptor domain. Amino acids 517 to 536 constitute the transmembrane region. Amino acids 537 to 966 constitute the intracellular catalytic domain. (See SEQ. ID. NOS. 3-4)

Figure 2.1 through 2.9 shows the cDNA and amino acid sequences of murine flk-1. All subsequent references to Figure 2

are intended to refer to Figure 2.1 through 2.9. Amino acids -  
19 to -1 constitute the hydrophobic leader sequence. Amino acids  
1 to 743 constitute the extracellular receptor domain. Amino  
acids 744 to 765 constitute the transmembrane region. Amino  
acids 766 to 1348 constitute the intracellular catalytic domain.  
(See SEQ. ID. NOS. 5-6)

Figure 3 shows the time response of binding between a murine  
stromal cell line (2018) and APTag-flk-2 as well as APTag-flk-1.  
APTtag without receptor (SEAP) is used as a control. See Example  
8.

Figure 4 shows the dose response of binding between stromal  
cells (2018) and APTag-flk-2 as well as APTag-flk-1. APTag  
without receptor (SEAP) is used as a control. See Example 8.

## DETAILED DESCRIPTION OF THE INVENTION

### Receptors

In one embodiment, the invention relates to an isolated  
mammalian nucleic acid molecule encoding a receptor protein  
tyrosine kinase expressed in primitive mammalian hematopoietic  
cells and not expressed in mature hematopoietic cells.

The nucleic acid molecule may be a DNA, cDNA, or RNA  
molecule. The mammal in which the nucleic acid molecule exists  
may be any mammal, such as a mouse, rat, rabbit, or human.

The nucleic acid molecule encodes a protein tyrosine kinase  
(pTK). Members of the pTK family can be recognized by the  
conserved amino acid regions in the catalytic domains. Examples  
of pTK consensus sequences have been provided by Hanks et al. in  
Science 241, 42-52 (1988); see especially Figure 1 starting on

page 46 and by Wilks in Proc. Natl. Acad. Sci. USA 86, 1603-1607 (1989); see especially Figure 2 on page 1605. A methionine residue at position 205 in the conserved sequence WMAPES is characteristic of pTK's that are receptors.

The Hanks et al article identifies eleven catalytic sub-domains containing pTK consensus residues and sequences. The pTKs of the present invention will have most or all of these consensus residues and sequences.

Some particularly strongly conserved residues and sequences are shown in Table 1.

**TABLE 1**

Conserved Residues and Sequences in pTKs<sup>1</sup>

<u>Position<sup>2</sup></u>	<u>Residue or Sequence</u>	<u>Catalytic Domain</u>
50	G	I
52	G	I
57	V	I
70	A	II
72	K	II
91	E	III
166	D	VI
171	N	VI
184-186	DFG	VII
208	E	VIII
220	D	IX
225	G	IX
280	R	XI

1. See Hanks et al., Science 241, 42-52 (1988)

2. Adjusted in accordance with Hanks et al., Id.

A pTK of the invention may contain all thirteen of these highly conserved residues and sequences. As a result of natural or synthetic mutations, the pTKs of the invention may contain fewer than all thirteen strongly conserved residues and sequences, such as 11, 9, or 7 such sequences.



The receptors of the invention generally belong to the same class of pTK sequences that c-kit belongs to. It has surprisingly been discovered, however, that a new functional class of receptor pTKs exists. The new functional class of receptor pTKs is expressed in primitive hematopoietic cells, but not expressed in mature hematopoietic cells.

For the purpose of this specification, a primitive hematopoietic cell is totipotent, i.e. capable of reconstituting all hematopoietic blood cells in vivo. A mature hematopoietic cell is non-self-renewing, and has limited proliferative capacity - i.e., a limited ability to give rise to multiple lineages. Mature hematopoietic cells, for the purposes of this specification, are generally capable of giving rise to only one or two lineages in vitro or in vivo.

It should be understood that the hematopoietic system is complex, and contains many intermediate cells between the primitive totipotent hematopoietic stem cell and the totally committed mature hematopoietic cells defined above. As the stem cell develops into increasingly mature, lineage-restricted cells, it gradually loses its capacity for self-renewal.

The receptors of the present invention may and may not be expressed in these intermediate cells. The necessary and sufficient condition that defines members of the new class of receptors is that they are present in the primitive, totipotent stem cell or cells, and not in mature cells restricted only to one or, at most, two lineages.

An example of a member of the new class of receptor pTKs is called fetal liver kinase 2 (flk-2) after the organ in which it was found. There is approximately 1 totipotent stem cell per  $10^4$  cells in mid-gestation (day 14) fetal liver in mice. In addition to fetal liver, flk-2 is also expressed in fetal spleen, fetal

thymus, adult brain, and adult marrow.

For example, flk-2 is expressed in individual multipotential CFU-Blast colonies capable of generating numerous multilineage colonies upon replating. It is likely, therefore, that flk-2 is expressed in the entire primitive (i.e. self-renewing) portion of the hematopoietic hierarchy. This discovery is consistent with flk-2 being important in transducing putative self-renewal signals from the environment.

It is particularly relevant that the expression of flk-2 mRNA occurs in the most primitive thymocyte subset. Even in two closely linked immature subsets that differ in expression of the IL-2 receptor, flk-2 expression segregates to the more primitive subset lacking an IL-2 receptor. The earliest thymocyte subset is believed to be uncommitted. Therefore, the thymocytes expressing flk-2 may be multipotential. flk-2 is the first receptor tyrosine kinase known to be expressed in the T-lymphoid lineage.

The fetal liver mRNA migrates relative to 28S and 18S ribosomal bands on formaldehyde agarose gels at approximately 3.5 kb, while the brain message is considerably larger. In adult tissues, flk-2 m-RNA from both brain and bone marrow migrated at approximately 3.5 kb.

A second pTK receptor is also included in the present invention. This second receptor, which is called fetal liver kinase 1 (flk-1), is not a member of the same class of receptors as flk-2, since flk-1 may be found in some more mature hematopoietic cells. The amino acid sequence of murine flk-1 is given in Figure 2.

The present invention includes the flk-1 receptor as well as DNA, cDNA and RNA encoding flk-1. The DNA sequence of murine

flk-1 is also given in Figure 2. Flk-1 may be found in the same organs as flk-2, as well as in fetal brain, stomach, kidney, lung, heart and intestine; and in adult kidney, heart, spleen, lung, muscle, and lymph nodes.

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The receptor protein tyrosine kinases of the invention are known to be divided into easily found domains. The DNA sequence corresponding to the pTKs encode, starting at their 5'-ends, a hydrophobic leader sequence followed by a hydrophilic extracellular domain, which binds to, and is activated by, a specific ligand. Immediately downstream from the extracellular receptor domain, is a hydrophobic transmembrane region. The transmembrane region is immediately followed by a basic catalytic domain, which may easily be identified by reference to the Hanks et al. and Wilks articles discussed above.

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09919408-073101  
154620-8046T650

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The following table shows the nucleic acid and amino acid numbers that correspond to the signal peptide, the extracellular domain, the transmembrane region and the intracellular domain for murine flk-1 (mflk-1), murine flk-2 (mflk-2) and human flk-2 (hflk-2).

#### mFLK-1

	<u>Signal Peptide</u>	<u>Extracellular</u>	<u>Transmembrane</u>	<u>Intracellular</u>
25	aa # -19 to -1	1 to 743	744 to 765	766 to 1348
	aa code M A	A E	V V	R A
	na # 208-264	265-2493	2494-2559	2560-4308

#### mFLK-2

	<u>Signal Peptide</u>	<u>Extracellular</u>	<u>Transmembrane</u>	<u>Intracellular</u>
30	aa # -27 to -1	1 to 517	518 to 537	538 to 966
	aa code M T	N S	F C	H S
	na # 31-111	112-1662	1663-1722	1723-3006

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## hFLK-2

### Signal Peptide Extracellular Transmembrane Intracellular

aa #	-27 to -1	1 to 516	517 to 536	537 to 966
aa code	M N	Q F	Y C	H S
na #	58-138	139-1689	1690-1746	1747-3036

The present invention includes the extracellular receptor domain lacking the transmembrane region and catalytic domain. Preferably, the hydrophobic leader sequence is also removed from the extracellular domain. In the case of human and murine flk-2, the hydrophobic leader sequence includes amino acids -27 to -1.

These regions and domains may easily be visually identified by those having ordinary skill in the art by reviewing the amino acid sequence in a suspected pTK and comparing it to known pTKs. For example, referring to Figure 1a, the transmembrane region of flk-2, which separates the extracellular receptor domain from the catalytic domain, is encoded by nucleotides 1663 (T) to 1722 (C). These nucleotides correspond to amino acid residues 545 (Phe) to 564 (Cys). The amino acid sequence between the transmembrane region and the catalytic sub-domain (amino acids 618-623) identified by Hanks et al. as sub-domain I (i.e., GXGXXG) is characteristic of receptor protein tyrosine kinases.

The extracellular domain may also be identified through commonly recognized criteria of extracellular amino acid sequences. The determination of appropriate criteria is known to those skilled in the art, and has been described, for example, by Hopp et al, Proc. Nat'l Acad. Sci. USA 78, 3824-3828 (1981); Kyte et al, J. Mol. Biol. 157, 105-132 (1982); Emini, J. Virol. 55, 836-839 (1985); Jameson et al, CA BIOS 4, 181-186 (1988); and Karplus et al, Naturwissenschaften 72, 212-213 (1985). Amino acid domains predicted by these criteria to be surface exposed characteristic of extracellular domains.

As will be discussed in more detail below, the nucleic acid molecules that encode the receptors of the invention may be inserted into known vectors for use in standard recombinant DNA techniques. Standard recombinant DNA techniques are those such as are described in Sambrook et al., "Molecular Cloning," Second Edition, Cold Spring Harbor Laboratory Press (1987) and by Ausubel et al., Eds, "Current Protocols in Molecular Biology," Green Publishing Associates and Wiley-Interscience, New York (1987). The vectors may be circular (i.e. plasmids) or non-circular. Standard vectors are available for cloning and expression in a host. The host may be prokaryotic or eucaryotic. Prokaryotic hosts are preferably E. coli. Preferred eucaryotic hosts include yeast, insect and mammalian cells. Preferred mammalian cells include, for example, CHO, COS and human cells.

### Ligands

The invention also includes ligands that bind to the receptor pTKs of the invention. In addition to binding, the ligands stimulate the proliferation of additional primitive stem cells, differentiation into more mature progenitor cells, or both.

The ligand may be a growth factor that occurs naturally in a mammal, preferably the same mammal that produces the corresponding receptor. The growth factor may be isolated and purified, or be present on the surface of an isolated population of cells, such as stromal cells.

The ligand may also be a molecule that does not occur naturally in a mammal. For example, antibodies, preferably monoclonal, raised against the receptors of the invention or against anti-ligand antibodies mimic the shape of, and act as, ligands if they constitute the negative image of the receptor or anti-ligand antibody binding site. The ligand may also be a non-

protein molecule that acts as a ligand when it binds to, or otherwise comes into contact with, the receptor. •

5 In another embodiment, nucleic acid molecules encoding the ligands of the invention are provided. The nucleic acid molecule may be RNA, DNA or cDNA.

### Stimulating Proliferation of Stem Cells

10 The invention also includes a method of stimulating the proliferation and/or differentiation of primitive mammalian hematopoietic stem cells as defined above. The method comprises contacting the stem cells with a ligand in accordance with the present invention. The stimulation of proliferation and/or differentiation may occur in vitro or in vivo.

15 The ability of a ligand according to the invention to stimulate proliferation of stem cells in vitro and in vivo has important therapeutic applications. Such applications include treating mammals, including humans, whose primitive stem cells do not sufficiently undergo self-renewal. Example of such medical problems include those that occur when defects in hematopoietic stem cells or their related growth factors depress the number of white blood cells. Examples of such medical problems include  
25 anemia, such as macrocytic and aplastic anemia. Bone marrow damage resulting from cancer chemotherapy and radiation is another example of a medical problem that would be helped by the stem cell factors of the invention.

### Functional Equivalents

30 The invention includes functional equivalents of the pTK receptors, receptor domains, and ligands described above as well as of the nucleic acid sequences encoding them. A protein is  
35 considered a functional equivalent of another protein for a

specific function if the equivalent protein is immunologically cross-reactive with, and has the same function as, the receptors and ligands of the invention. The equivalent may, for example, be a fragment of the protein, or a substitution, addition or deletion mutant of the protein.

For example, it is possible to substitute amino acids in a sequence with equivalent amino acids. Groups of amino acids known normally to be equivalent are:

- (a)Ala(A) Ser(S) Thr(T) Pro(P) Gly(G);
- (b)Asn(N) Asp(D) Glu(E) Gln(Q);
- (c)His(H) Arg(R) Lys(K);
- (d)Met(M) Leu(L) Ile(I) Val(V); and
- (e)Phe(F) Tyr(Y) Trp(W).

Substitutions, additions and/or deletions in the receptors and ligands may be made as long as the resulting equivalent receptors and ligands are immunologically cross reactive with, and have the same function as, the native receptors and ligands.

The equivalent receptors and ligands will normally have substantially the same amino acid sequence as the native receptors and ligands. An amino acid sequence that is substantially the same as another sequence, but that differs from the other sequence by means of one or more substitutions, additions and/or deletions is considered to be an equivalent sequence. Preferably, less than 25%, more preferably less than 10%, and most preferably less than 5% of the number of amino acid residues in the amino acid sequence of the native receptors and ligands are substituted for, added to, or deleted from.

Equivalent nucleic acid molecules include nucleic acid sequences that encode equivalent receptors and ligands as defined

above. Equivalent nucleic acid molecules also include nucleic acid sequences that differ from native nucleic acid sequences in ways that do not affect the corresponding amino acid sequences.

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## ISOLATION OF NUCLEIC ACID MOLECULES AND PROTEINS

### Isolation of Nucleic Acid Molecules Encoding Receptors

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In order to produce nucleic acid molecules encoding mammalian stem cell receptors, a source of stem cells is provided. Suitable sources include fetal liver, spleen, or thymus cells or adult marrow or brain cells.

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For example, suitable mouse fetal liver cells may be obtained at day 14 of gestation. Mouse fetal thymus cells may be obtained at day 14-18, preferably day 15, of gestation. Suitable fetal cells of other mammals are obtained at gestation times corresponding to those of mouse.

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Total RNA is prepared by standard procedures from stem cell receptor-containing tissue. The total RNA is used to direct cDNA synthesis. Standard methods for isolating RNA and synthesizing cDNA are provided in standard manuals of molecular biology such as, for example, in Sambrook et al., "Molecular Cloning," Second Edition, Cold Spring Harbor Laboratory Press (1987) and in Ausubel et al., (Eds), "Current Protocols in Molecular Biology," Greene Associates/Wiley Interscience, New York (1990).

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The cDNA of the receptors is amplified by known methods. For example, the cDNA may be used as a template for amplification by polymerase chain reaction (PCR); see Saiki et al., Science, 239, 487 (1988) or Mullis et al., U.S. patent 4,683,195. The sequences of the oligonucleotide primers for the PCR amplification are derived from the sequences of known receptors, such as from the sequences given in Figures 1 and 2 for flk-2 and



flk-1, respectively, preferably from flk-2. The oligonucleotides are synthesized by methods known in the art. Suitable methods include those described by Caruthers in Science 230, 281-285 (1985).

5

In order to isolate the entire protein-coding regions for the receptors of the invention, the upstream oligonucleotide is complementary to the sequence at the 5' end, preferably encompassing the ATG start codon and at least 5-10 nucleotides upstream of the start codon. The downstream oligonucleotide is complementary to the sequence at the 3' end, optionally encompassing the stop codon. A mixture of upstream and downstream oligonucleotides are used in the PCR amplification. The conditions are optimized for each particular primer pair according to standard procedures. The PCR product is analyzed by electrophoresis for the correct size cDNA corresponding to the sequence between the primers.

Alternatively, the coding region may be amplified in two or more overlapping fragments. The overlapping fragments are designed to include a restriction site permitting the assembly of the intact cDNA from the fragments.

The amplified DNA encoding the receptors of the invention may be replicated in a wide variety of cloning vectors in a wide variety of host cells. The host cell may be prokaryotic or eukaryotic. The DNA may be obtained from natural sources and, optionally, modified, or may be synthesized in whole or in part.

The vector into which the DNA is spliced may comprise segments of chromosomal, non-chromosomal and synthetic DNA sequences. Some suitable prokaryotic cloning vectors include plasmids from E. coli, such as colE1, pCR1, pBR322, pMB9, pUC, pKSM, and RP4. Prokaryotic vectors also include derivatives of phage DNA such as M13 and other filamentous single-stranded DNA

phages.

### Isolation of Receptors

5 DNA encoding the receptors of the invention are inserted  
into a suitable vector and expressed in a suitable prokaryotic or  
eucaryotic host. Vectors for expressing proteins in bacteria,  
especially E.coli, are known. Such vectors include the PATH  
vectors described by Dieckmann and Tzagoloff in J. Biol. Chem.  
10 260, 1513-1520 (1985). These vectors contain DNA sequences that  
encode anthranilate synthetase (TrpE) followed by a polylinker at  
the carboxy terminus. Other expression vector systems are based  
on beta-galactosidase (pEX); lambda P<sub>L</sub>; maltose binding protein  
(pMAL); and glutathione S-transferase (pGST) - see Gene 67, 31  
(1988) and Peptide Research 3, 167 (1990).

Vectors useful in yeast are available. A suitable example  
is the 2μ plasmid.

20 Suitable vectors for use in mammalian cells are also known.  
Such vectors include well-known derivatives of SV-40, adenovirus,  
retrovirus-derived DNA sequences and shuttle vectors derived from  
combination of functional mammalian vectors, such as those  
described above, and functional plasmids and phage DNA.

25 Further eukaryotic expression vectors are known in the art  
(e.g., P.J. Southern and P. Berg, J. Mol. Appl. Genet. 1, 327-341  
(1982); S. Subramani et al, Mol. Cell. Biol. 1, 854-864 (1981);  
R.J. Kaufmann and P.A. Sharp, "Amplification And Expression Of  
30 Sequences Cotransfected with A Modular Dihydrofolate Reductase  
Complementary DNA Gene," J. Mol. Biol. 159, 601-621 (1982); R.J.  
Kaufmann and P.A. Sharp, Mol. Cell. Biol. 159, 601-664 (1982);  
S.I. Scahill et al, "Expression And Characterization Of The  
Product Of A Human Immune Interferon DNA Gene In Chinese Hamster  
35 Ovary Cells," Proc. Natl. Acad. Sci. USA 80, 4654-4659 (1983); G.

Urlaub and L.A. Chasin, Proc. Natl. Acad. Sci. USA 77, 4216-4220, (1980).

0593408-073101  
10 20 30 40 50 60 70 80 90 100 110 120 130 140 150 160 170 180 190 200 210 220 230 240 250 260 270 280 290 300 310 320 330 340 350 360 370 380 390 400 410 420 430 440 450 460 470 480 490 500 510 520 530 540 550 560 570 580 590 600 610 620 630 640 650 660 670 680 690 700 710 720 730 740 750 760 770 780 790 800 810 820 830 840 850 860 870 880 890 900 910 920 930 940 950 960 970 980 990 1000

The expression vectors useful in the present invention contain at least one expression control sequence that is operatively linked to the DNA sequence or fragment to be expressed. The control sequence is inserted in the vector in order to control and to regulate the expression of the cloned DNA sequence. Examples of useful expression control sequences are the lac system, the trp system, the tac system, the trc system, major operator and promoter regions of phage lambda, the control region of fd coat protein, the glycolytic promoters of yeast, e.g., the promoter for 3-phosphoglycerate kinase, the promoters of yeast acid phosphatase, e.g., Pho5, the promoters of the yeast alpha-mating factors, and promoters derived from polyoma, adenovirus, retrovirus, and simian virus, e.g., the early and late promoters of SV40, and other sequences known to control the expression of genes of prokaryotic or eukaryotic cells and their viruses or combinations thereof.

Vectors containing the receptor-encoding DNA and control signals are inserted into a host cell for expression of the receptor. Some useful expression host cells include well-known prokaryotic and eukaryotic cells. Some suitable prokaryotic hosts include, for example, E. coli, such as E. coli SG-936, E. coli HB 101, E. coli W3110, E. coli X1776, E. coli X2282, E. coli DHI, and E. coli MRC1, Pseudomonas, Bacillus, such as Bacillus subtilis, and Streptomyces. Suitable eukaryotic cells include yeast and other fungi, insect, animal cells, such as COS cells and CHO cells, human cells and plant cells in tissue culture.

The human homologs of the mouse receptors described above are isolated by a similar strategy. RNA encoding the receptors are obtained from a source of human cells enriched for primitive stem cells. Suitable human cells include fetal spleen, thymus

and liver cells, and umbilical cord blood as well as adult brain and bone marrow cells. The human fetal cells are preferably obtained on the day of gestation corresponding to mid-gestation in mice. The amino acid sequences of the human flk receptors as well as of the nucleic acid sequences encoding them are homologous to the amino acid and nucleotide sequences of the mouse receptors.

In the present specification, the sequence of a first protein, such as a receptor or a ligand, or of a nucleic acid molecule that encodes the protein, is considered homologous to a second protein or nucleic acid molecule if the amino acid or nucleotide sequence of the first protein or nucleic acid molecule is at least about 30% homologous, preferably at least about 50% homologous, and more preferably at least about 65% homologous to the respective sequences of the second protein or nucleic acid molecule. In the case of proteins having high homology, the amino acid or nucleotide sequence of the first protein or nucleic acid molecule is at least about 75% homologous, preferably at least about 85% homologous, and more preferably at least about 95% homologous to the amino acid or nucleotide sequence of the second protein or nucleic acid molecule.

Combinations of mouse oligonucleotide pairs are used as PCR primers to amplify the human homologs from the cells to account for sequence divergence. The remainder of the procedure for obtaining the human flk homologs are similar to those described above for obtaining mouse flk receptors. The less than perfect homology between the human flk homologs and the mouse oligonucleotides is taken into account in determining the stringency of the hybridization conditions.

#### **Assay for expression of Receptors on Stem Cells**

In order to demonstrate the expression of flk receptors on

the surface of primitive hematopoietic stem cells, antibodies that recognize the receptor are raised. The receptor may be the entire protein as it exists in nature, or an antigenic fragment of the whole protein. Preferably, the fragment comprises the predicted extra-cellular portion of the molecule.

Antigenic fragments may be identified by methods known in the art. Fragments containing antigenic sequences may be selected on the basis of generally accepted criteria of potential antigenicity and/or exposure. Such criteria include the hydrophilicity and relative antigenic index, as determined by surface exposure analysis of proteins. The determination of appropriate criteria is known to those skilled in the art, and has been described, for example, by Hopp et al, Proc. Nat'l Acad. Sci. USA 78, 3824-3828 (1981); Kyte et al, J. Mol. Biol. 157, 105-132 (1982); Emini, J. Virol. 55, 836-839 (1985); Jameson et al, CA BIOS 4, 181-186 (1988); and Karplus et al, Naturwissenschaften 72, 212-213 (1985). Amino acid domains predicted by these criteria to be surface exposed are selected preferentially over domains predicted to be more hydrophobic or hidden.

The proteins and fragments of the receptors to be used as antigens may be prepared by methods known in the art. Such methods include isolating or synthesizing DNA encoding the proteins and fragments, and using the DNA to produce recombinant proteins, as described above.

Fragments of proteins and DNA encoding the fragments may be chemically synthesized by methods known in the art from individual amino acids and nucleotides. Suitable methods for synthesizing protein fragments are described by Stuart and Young in "Solid Phase Peptide Synthesis," Second Edition, Pierce Chemical Company (1984). Suitable methods for synthesizing DNA fragments are described by Caruthers in Science 230, 281-285

(1985).

If the receptor fragment defines the epitope, but is too short to be antigenic, it may be conjugated to a carrier molecule in order to produce antibodies. Some suitable carrier molecules include keyhole limpet hemocyanin, Ig sequences, TrpE, and human or bovine serum albumen. Conjugation may be carried out by methods known in the art. One such method is to combine a cysteine residue of the fragment with a cysteine residue on the carrier molecule.

The antibodies are preferably monoclonal. Monoclonal antibodies may be produced by methods known in the art. These methods include the immunological method described by Kohler and Milstein in *Nature* 256, 495-497 (1975) and Campbell in "Monoclonal Antibody Technology, The Production and Characterization of Rodent and Human Hybridomas" in Burdon et al., Eds, *Laboratory Techniques in Biochemistry and Molecular Biology*, Volume 13, Elsevier Science Publishers, Amsterdam (1985); as well as by the recombinant DNA method described by Huse et al in *Science* 246, 1275-1281 (1989).

Polyclonal or monoclonal antisera shown to be reactive with receptor-encoded native proteins, such as with flk-1 and flk-2 encoded proteins, expressed on the surface of viable cells are used to isolate antibody-positive cells. One method for isolating such cells is flow cytometry; see, for example, Loken et al., European patent application 317,156. The cells obtained are assayed for stem cells by engraftment into radiation-ablated hosts by methods known in the art; see, for example, Jordan et al., *Cell* 61, 953-963 (1990).

**Criteria for Novel Stem Cell Receptor Tyrosine Kinases  
Expressed in Stem Cells**

Additional novel receptor tyrosine kinase cDNAs are obtained  
5 by amplifying cDNAs from stem cell populations using  
oligonucleotides as PCR primers; see above. Examples of suitable  
oligonucleotides are PTK1 and PTK2, which were described by Wilks  
et al. in Proc. Natl. Acad. Sci. USA 86, 1603-1607 (1989). Novel  
10 cDNA is selected on the basis of differential hybridization  
screening with probes representing known kinases. The cDNA  
clones hybridizing only at low stringency are selected and  
sequenced. The presence of the amino acid triplet DFG confirms  
that the sequence represents a kinase. The diagnostic methionine  
15 residue in the WMAPES motif is indicative of a receptor-like  
kinase, as described above. Potentially novel sequences obtained  
are compared to available sequences using databases such as  
Genbank in order to confirm uniqueness. Gene-specific  
oligonucleotides are prepared as described above based on the  
20 sequence obtained. The oligonucleotides are used to analyze stem  
cell enriched and depleted populations for expression. Such cell  
populations in mice are described, for example, by Jordan et al.  
in Cell 61, 953-956 (1990); Ikuta et al. in Cell 62, 863-864  
(1990); Spangrude et al. in Science 241, 58-62 (1988); and  
Szilvassy et al. in Blood 74, 930-939 (1989). Examples of such  
25 human cell populations are described as CD33<sup>+</sup>CD34<sup>+</sup> by Andrews et  
al. in the Journal of Experimental Medicine 169, 1721-1731  
(1989). Other human stem cell populations are described, for  
example, in Civin et al., European Patent Application 395,355 and  
30 in Loken et al., European Patent Application 317,156.

**Isolating Ligands and Nucleic Acid Molecules Encoding Ligands**

35 Cells that may be used for obtaining ligands include stromal  
cells, for example stromal cells from fetal liver, fetal spleen,

fetal thymus and fetal or adult bone marrow. Cell lines expressing ligands are established and screened.

For example, cells such as stromal (non-hematopoietic) cells from fetal liver are immortalized by known methods. Examples of known methods of immortalizing cells include transduction with a temperature sensitive SV40 T-antigen expressed in a retroviral vector. Infection of fetal liver cells with this virus permits the rapid and efficient establishment of multiple independent cell lines. These lines are screened for ligand activity by methods known in the art, such as those outlined below.

Ligands for the receptors of the invention, such as flk-1 and flk-2, may be obtained from the cells in several ways. For example, a bioassay system for ligand activity employs chimeric tagged receptors; see, for example, Flanagan et al., Cell 63, 185-194 (1990). One strategy measures ligand binding directly via a histochemical assay. Fusion proteins comprising the extracellular receptor domains and secretable alkaline phosphatase (SEAP) are constructed and transfected into suitable cells such as NIH/3T3 or COS cells. Flanagan et al. refer to such DNA or amino acid constructs as APTag followed by the name of the receptor - i.e. APTag-c-kit. The fusion proteins bind with high affinity to cells expressing surface-bound ligand. Binding is detectable by the enzymatic activity of the alkaline phosphatase secreted into the medium. The bound cells, which are often stromal cells, are isolated from the APTag-receptor complex.

For example, some stromal cells that bind APTag-flk1 and APTag-flk2 fusion proteins include mouse fetal liver cells (see example 1); human fetal spleen cells (see example 3); and human fetal liver (example 3). Some stromal fetal thymus cells contain flk-1 ligand (example 3).



To clone the cDNA that encodes the ligand, a cDNA library is constructed from the isolated stromal cells in a suitable expression vector, preferably a phage such as CDM8, pSV Sport (BRL Gibco) or piH3, (Seed et al., Proc. Natl. Acad. Sci. USA 84, 3365-3369 (1987)). The library is transfected into suitable host cells, such as COS cells. Cells containing ligands on their surface are detected by known methods, see above.

In one such method, transfected COS cells are distributed into single cell suspensions and incubated with the secreted alkaline phosphatase-flk receptor fusion protein, which is present in the medium from NIH/3T3 or COS cells prepared by the method described by Flanagan et al., see above. Alkaline phosphatase-receptor fusion proteins that are not bound to the cells are removed by centrifugation, and the cells are panned on plates coated with antibodies to alkaline phosphatase. Bound cells are isolated following several washes with a suitable wash reagent, such as 5% fetal bovine serum in PBS, and the DNA is extracted from the cells. Additional details of the panning method described above may be found in an article by Seed et al., Proc. Natl. Acad. Sci. USA 84, 3365-3369 (1987).

In a second strategy, the putative extracellular ligand binding domains of the receptors are fused to the transmembrane and kinase domains of the human c-fms tyrosine kinase and introduced into 3T3 fibroblasts. The human c-fms kinase is necessary and sufficient to transduce proliferative signals in these cells after appropriate activation i.e. with the flk-1 or flk-2 ligand. The 3T3 cells expressing the chimeras are used to screen putative sources of ligand in a cell proliferation assay.

An alternate approach for isolating ligands using the fusion receptor-expressing 3T3 cells and insertional activation is also possible. A retrovirus is introduced into random chromosomal positions in a large population of these cells. In a small

fraction, the retrovirus is inserted in the vicinity of the ligand-encoding gene, thereby activating it. These cells proliferate due to autocrine stimulation of the receptor. The ligand gene is "tagged" by the retrovirus, thus facilitating its isolation.

### Examples

#### Example 1. Cells containing mouse flk-1 and flk-2 ligands. Murine stromal cell line 2018.

In order to establish stromal cell lines, fetal liver cells are disaggregated with collagen and grown in a mixture of Dulbecco's Modified Eagle's Medium (DMEM) and 10% heat-inactivated fetal calf serum at 37°C. The cells are immortalized by standard methods. A suitable method involves introducing DNA encoding a growth regulating- or oncogene-encoding sequence into the target host cell. The DNA may be introduced by means of transduction in a recombinant viral particle or transfection in a plasmid. See, for example, Hammerschmidt et al., Nature 340, 393-397 (1989) and Abcouwer et al, Biotechnology 7, 939-946 (1989). Retroviruses are the preferred viral vectors, although SV40 and Epstein-Barr virus can also serve as donors of the growth-enhancing sequences. A suitable retrovirus is the ecotropic retrovirus containing a temperature sensitive SV40 T-antigen (tsA58) and a G418 resistance gene described by McKay in Cell 66, 713-729 (1991). After several days at 37°C, the temperature of the medium is lowered to 32°C. Cells are selected with G418 (0.5 mg/ml). The selected cells are expanded and maintained.

A mouse stromal cell line produced by this procedure is called 2018 and was deposited on October 30, 1991 in the American Type Culture Collection, Rockville, Maryland, USA (ATCC);

accession number CRL 10907.

Example 2. Cells containing human flk-1 and flk-2 ligands.

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Human fetal liver (18, 20, and 33 weeks after abortion), spleen (18 weeks after abortion), or thymus (20 weeks after abortion) is removed at the time of abortion and stored on ice in a balanced salt solution. After mincing into 1 mm fragments and forcing through a wire mesh, the tissue is washed one time in Hanks Balanced Salt Solution (HBSS).

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The disrupted tissue is centrifuged at 200 xg for 15 minutes at room temperature. The resulting pellet is resuspended in 10-20 ml of a tissue culture grade trypsin-EDTA solution (Flow Laboratories). The resuspended tissue is transferred to a sterile flask and stirred with a stirring bar at room temperature for 10 minutes. One ml of heat-inactivated fetal bovine calf serum (Hyclone) is added to a final concentration of 10% in order to inhibit trypsin activity. Collagenase type IV (Sigma) is added from a stock solution (10 mg/ml in HBSS) to a final concentration of 100 ug/ml in order to disrupt the stromal cells. The tissue is stirred at room temperature for an additional 2.5 hours; collected by centrifugation (400xg, 15 minutes); and resuspended in "stromal medium," which contains Iscove's modification of DMEM supplemented with 10% heat-inactivated fetal calf serum, 5% heat-inactivated human serum (Sigma), 4 mM L-glutamine, 1x sodium pyruvate, (stock of 100x Sigma), 1x non-essential amino acids (stock of 100x, Flow), and a mixture of antibiotics kanomycin, neomycin, penicillin, streptomycin. Prior to resuspending the pellet in the stromal medium, the pellet is washed one time with HBSS. It is convenient to suspend the cells in 60 ml of medium. The number of cultures depends on the amount of tissue.

### Example 3. Isolating Stromal cells

Resuspended Cells (example 2) that are incubated at 37°C with 5% carbon dioxide begin to adhere to the plastic plate within 10-48 hours. Confluent monolayers may be observed within 7-10 days, depending upon the number of cells plated in the initial inoculum. Non-adherent and highly refractile cells adhering to the stromal cell layer as colonies are separately removed by pipetting and frozen. Non-adherent cells are likely sources of populations of self-renewing stem cells containing flk-2. The adherent stromal cell layers are frozen in aliquots for future studies or expanded for growth in culture.

An unexpectedly high level of APTag-flk-2 fusion protein binding to the fetal spleen cells is observed. Two fetal spleen lines are grown in "stromal medium," which is described in example 2.

Non-adherent fetal stem cells attach to the stromal cells and form colonies (colony forming unit - CFU). Stromal cells and CFU are isolated by means of sterile glass cylinders and expanded in culture. A clone, called Fsp 62891, contains the flk-2 ligand. Fsp 62891 was deposited in the American Type Culture Collection, Rockville, Maryland, U.S.A on November 21, 1991, accession number CRL 10935.

Fetal liver and fetal thymus cells are prepared in a similar way. Both of these cell types produce ligands of flk-1 and, in the case of liver, some flk-2. One such fetal thymus cell line, called F.thy 62891, and one such fetal liver cell line, called FL 62891, were deposited in the American Type Culture Collection, Rockville, Maryland, U.S.A on November 21, 1991 and April 2, 1992, respectively, accession numbers CRL 10936 and CRL 11005, respectively.

Stable human cell lines are prepared from fetal cells with the same temperature sensitive immortalizing virus used to prepare the murine cell line described in example 1.

5 **Example 4. Isolation of human stromal cell clone**

Highly refractile cells overgrow patches of stromal cells, presumably because the stromal cells produce factors that allow the formation of the CFU. To isolate stromal cell clones,  
10 sterile glass cylinders coated with vacuum grease are positioned over the CFU. A trypsin-EDTA solution (100 ml) is added in order to detach the cells. The cells are added to 5 ml of stromal medium and each (clone) plated in a single well of 6-well plate.

15 **Example 5. Plasmid (AP-tag) for expressing secretable alkaline phosphatase (SEAP)**

Plasmids that express secretable alkaline phosphatase are described by Flanagan and Leder in Cell 63, 185-194 (1990). The plasmids contain a promoter, such as the LTR promoter; a polylinker, including HindIII and BglII; DNA encoding SEAP; a poly-A signal; and ampicillin resistance gene; and replication site.

25 **Example 6. Plasmid for expressing APTag-flk-2 and APTag-flk-1 fusion proteins**

Plasmids that express fusion proteins of SEAP and the extracellular portion of either flk-1 or flk-2 are prepared in accordance with the protocols of Flanagan and Leder in Cell 63, 185-194 (1990) and Berger et al., Gene 66, 1-10 (1988). Briefly,  
30 a HindIII-Bam HI fragment containing the extracellular portion of flk-1 or flk-2 is prepared and inserted into the HindIII-BglII  
35 site of the plasmid described in example 5.

### Example 7. Production Of APtag-flk-1 Or -flk-2 Fusion Protein

5 The plasmids from Example 6 are transfected into Cos-7 cells  
by DEAE-dextran (as described in Current Protocols in Molecular  
Biology, Unit 16.13, "Transient Expression of Proteins Using Cos  
Cells," 1991); and cotransfected with a selectable marker, such  
as pSV7neo, into NIH/3T3 cells by calcium precipitation. The  
10 NIH/3T3 cells are selected with 600µg/ml G418 in 100 mm plates.  
Over 300 clones are screened for secretion of placental alkaline  
phosphatase activity. The assay is performed by heating a  
portion of the supernatant at 65°C for 10 minutes to inactivate  
background phosphatase activity, and measuring the OD<sub>405</sub> after  
incubating with 1M diethanolamine (pH 9.8), 0.5 mM MgCl<sub>2</sub>, 10 mM  
L-homoarginine (a phosphatase inhibitor), 0.5 mg/ml BSA, and 12  
mM p-nitrophenyl phosphate. Human placental alkaline phosphatase  
is used to perform a standard curve. The APtag-flk-1 clones (F-  
1AP21-4) produce up to 10 µg alkaline phosphatase activity/ml and  
the APtag-flk-2 clones (F-2AP26-0) produce up to 0.5 µg alkaline  
phosphatase activity/ml.

### Example 8. Assay For APtag-flk-1 Or APtag-flk-2 Binding To Cells

25 The binding of APtag-flk-1 or APtag-flk-2 to cells  
containing the appropriate ligand is assayed by standard methods.  
See, for example, Flanagan and Leder, Cell 63:185-194, 1990).  
Cells (i.e., mouse stromal cells, human fetal liver, spleen or  
30 thymus, or various control cells) are grown to confluency in six-  
well plates and washed with HBHA (Hank's balanced salt solution  
with 0.5 mg/ml BSA, 0.02% NaN<sub>3</sub>, 20 mM HEPES, pH 7.0).  
Supernatants from transfected COS or NIH/3T3 cells containing  
either APtag-flk-1 fusion protein, APtag-flk-2 fusion protein, or  
35 APtag without a receptor (as a control) are added to the cell  
monolayers and incubated for two hours at room temperature on a  
rotating platform. The concentration of the APtag-flk-1 fusion

protein, APTag-flk-2 fusion protein, or APTag without a receptor is 60 ng/ml of alkaline phosphatase as determined by the standard alkaline phosphatase curve (see above). The cells are then rinsed seven times with HBHA and lysed in 350  $\mu$ l of 1% Triton X-100, 10 mM Tris-HCl (pH 8.0). The lysates are transferred to a microfuge tube, along with a further 150  $\mu$ l rinse with the same solution. After vortexing vigorously, the samples are centrifuged for five minutes in a microfuge, heated at 65°C for 12 minutes to inactivate cellular phosphatases, and assayed for phosphatase activity as described previously. Results of experiments designed to show the time and dose responses of binding between stromal cells containing the ligands to flk-2 and flk-1 (2018) and APTag-flk-2, APTag-flk-1 and APTag without receptor (as a control) are shown in Figures 3 and 4, respectively.

Example 8A. Plasmids for expressing flk1/fms and flk2/fms fusion proteins

Plasmids that express fusion proteins of the extracellular portion of either flk-1 or flk-2 and the intracellular portion of c-fms (also known as colony-stimulating factor-1 receptor) are prepared in a manner similar to that described under Example 6 (Plasmid for expressing APTag-flk-2 and APTag-flk-1 fusion proteins). Briefly, a Hind III - Bam HI fragment containing the extracellular portion of flk1 or flk2 is prepared and inserted into the Hind III - Bgl II site of a pLH expression vector containing the intracellular portion of c-fms.

8B. Expression of flk1/fms or flk2/fms in 3T3 cells

The plasmids from Example 11 are transfected into NIH/3T3 cells by calcium. The intracellular portion of c-fms is detected by Western blotting.

Example 9. Cloning and Expression of cDNA Coding For Mouse  
Ligand To flk-1 and flk-2 Receptors

5 cDNA expressing mouse ligand for flk-1 and flk-2 is prepared  
by known methods. See, for example, Seed, B., and Aruffo, A.  
PNAS 84:3365-3369, 1987; Simmons, D. and Seed, B. J. Immunol.  
141:2797-2800; and D'Andrea, A.D., Lodish, H.F. and Wong, G.G.  
Cell 57:277-285, 1989).

10 The protocols are listed below in sequence: (a) RNA  
isolation; (b) poly A RNA preparation; (c) cDNA synthesis; (d)  
cDNA size fractionation; (e) propagation of plasmids (vector);  
15 (f) isolation of plasmid DNA; (g) preparation of vector pSV Sport  
(BRL Gibco) for cloning; (h) compilation of buffers for the above  
steps; (i) Transfection of cDNA encoding Ligands in Cos 7 Cells;  
(j) panning procedure; (k) Expression cloning of flk-1 or flk-2  
ligand by establishment of an autocrine loop.

20 9a. Guanidinium thiocyanate/LiCl Protocol for RNA Isolation

For each ml of mix desired, 0.5 g guanidine thiocyanate  
(GuSCN) is dissolved in 0.55 ml of 25% LiCl (stock filtered  
through 0.45 micron filter). 20 µl of mercaptoethanol is added.  
25 (The resulting solution is not good for more than about a week at  
room temperature.)

30 The 2018 stromal cells are centrifuged, and 1 ml of the  
solution described above is added to up to  $5 \times 10^7$  cells. The  
cells are sheared by means of a polytron until the mixture is  
non-viscous. For small scale preparations ( $<10^8$  cells), the  
sheared mixture is layered on 1.5 ml of 5.7M CsCl (RNase free;  
1.26 g CsCl added to every ml 10 mM EDTA pH8), and overlaid with  
RNase-free water if needed. The mixture is spun in an SW55 rotor  
35 at 50 krpm for 2 hours. For large scale preparations, 25 ml of  
the mixture is layered on 12 ml CsCl in an SW28 tube, overlaid as



above, and spun at 24 krpm for 8 hours. The contents of the tube are aspirated carefully with a sterile pasteur pipet connected to a vacuum flask. Once past the CsCl interface, a band around the tube is scratched with the pipet tip to prevent creeping of the layer on the wall down the tube. The remaining CsCl solution is aspirated. The resulting pellet is taken up in water, but not redissolved. 1/10 volume of sodium acetate and three volumes of ethanol are added to the mixture, and spun. The pellet is resuspended in water at 70°C, if necessary. The concentration of the RNA is adjusted to 1 mg/ml and frozen.

It should be noted that small RNA molecules (e.g., 5S) do not come down. For small amounts of cells, the volumes are scaled down, and the mixture is overlaid with GuSCN in RNase-free water on a gradient (precipitation is inefficient when RNA is dilute).

#### 9b. Poly A<sup>+</sup> RNA preparation

(All buffers mentioned are compiled separately below)

A disposable polypropylene column is prepared by washing with 5M NaOH and then rinsing with RNase-free water. For each milligram of total RNA, approximately 0.3 ml (final packed bed) of oligo dT cellulose is added. The oligo dT cellulose is prepared by resuspending approximately 0.5 ml of dry powder in 1 ml of 0.1M NaOH and transferring it into the column, or by percolating 0.1M NaOH through a previously used column. The column is washed with several column volumes of RNase-free water until the pH is neutral, and rinsed with 2-3 ml of loading buffer. The column bed is transferred to a sterile 15 ml tube using 4-6 ml of loading buffer.

Total RNA from the 2018 cell line is heated to 70°C for 2-3 minutes. LiCl from RNase-free stock is added to the mixture to a final concentration of 0.5M. The mixture is combined with oligo

dT cellulose in the 15 ml tube, which is vortexed or agitated for 10 minutes. The mixture is poured into the column, and washed with 3 ml loading buffer, and then with 3 ml of middle wash buffer. The mRNA is eluted directly into an SW55 tube with 1.5 ml of 2 mM EDTA and 0.1% SDS, discarding the first two or three drops.

The eluted mRNA is precipitated by adding 1/10 volume of 3M sodium acetate and filling the tube with ethanol. The contents of the tube are mixed, chilled for 30 minutes at -20°C, and spun at 50 krpm at 5°C for 30 minutes. After the ethanol is decanted, and the tube air dried, the mRNA pellet is resuspended in 50-100 µl of RNase-free water. 5 µl of the resuspended mRNA is heated to 70°C in MOPS/EDTA/formaldehyde, and examined on an RNase-free 1% agarose gel.

#### 9c. cDNA Synthesis

The protocol used is a variation of the method described by Gubler and Hoffman in Gene 25, 263-270 (1983).

1. First Strand. 4 µg of mRNA is added to a microfuge tube, heated to approximately 100°C for 30 seconds, quenched on ice. The volume is adjusted to 70µl with RNase-free water. 20 µl of RT1 buffer, 2 µl of RNase inhibitor (Boehringer 36 u/µl), 1 µl of 5 µg/µl of oligo dT (Collaborative Research), 2.5 µl of 20 mM dXTP's (ultrapure - US Biochemicals), 1 µl of 1M DTT and 4 µl of RT-XL (Life Sciences, 24 u/µl) are added. The mixture is incubated at 42°C for 40 minutes, and inactivated by heating at 70°C for 10 minutes.

2. Second Strand. 320 µl of RNase-free water, 80 µl of RT2 buffer, 5 µl of DNA Polymerase I (Boehringer, 5 U/µl), 2 µl RNase H (BRL 2 u/µl) are added to the solution containing the first strand. The solution is incubated at 15°C for one hour and at

22°C for an additional hour. After adding 20 µl of 0.5M EDTA, pH 8.0, the solution is extracted with phenol and precipitated by adding NaCl to 0.5M linear polyacrylamide (carrier) to 20 µg/ml, and filling the tube with EtOH. The tube is spun for 2-3 minutes in a microfuge, vortexed to dislodge precipitated material from the wall of the tube, and respun for one minute.

3. Adaptors. Adaptors provide specific restriction sites to facilitate cloning, and are available from BRL Gibco, New England Biolabs, etc. Crude adaptors are resuspended at a concentration of 1 µg/µl. MgSO<sub>4</sub> is added to a final concentration of 10 mM, followed by five volumes of EtOH. The resulting precipitate is rinsed with 70% EtOH and resuspended in TE at a concentration of 1 µg/µl. To kinase, 25 µl of resuspended adaptors is added to 3 µl of 10X kinasing buffer and 20 units of kinase. The mixture is incubated at 37°C overnight. The precipitated cDNA is resuspended in 240 µl of TE (10/1). After adding 30 µl of 10X low salt buffer, 30 µl of 10X ligation buffer with 0.1mM ATP, 3 µl (2.4 µg) of kinased 12-mer adaptor sequence, 2 µl (1.6 µg) of kinased 8-mer adaptor sequence, and 1 µl of T4 DNA ligase (BioLabs, 400 u/µl, or Boehringer, 1 Weiss unit ml), the mixture is incubated at 15°C overnight. The cDNA is extracted with phenol and precipitated as above, except that the extra carrier is omitted, and resuspended in 100 µl of TE.

#### 9d. cDNA Size Fractionation.

A 20% KOAc, 2 mM EDTA, 1 µg/ml ethidium bromide solution and a 5% KOAc, 2 mM EDTA, 1 µg/ml ethidium bromide solution are prepared. 2.6 ml of the 20% KOAc solution is added to the back chamber of a small gradient maker. Air bubbles are removed from the tube connecting the two chambers by allowing the 20% solution to flow into the front chamber and forcing the solution to return to the back chamber by tilting the gradient maker. The passage between the chambers is closed, and 2.5 ml of 5% solution is

added to the front chamber. Any liquid in the tubing from a previous run is removed by allowing the 5% solution to flow to the end of the tubing, and then to return to its chamber. The apparatus is placed on a stirplate, and, with rapid stirring, the topcock connecting the two chambers and the front stopcock are opened. A polyallomer SW55 tube is filled from the bottom with the KOAc solution. The gradient is overlaid with 100  $\mu$ l of cDNA solution, and spun for three hours at 50k rpm at 22°C. To collect fractions from the gradient, the SW55 tube is pierced close to the bottom of the tube with a butterfly infusion set (with the luer hub clipped off). Three 0.5 ml fractions and then six 0.25 ml fractions are collected in microfuge tubes (approximately 22 and 11 drops, respectively). The fractions are precipitated by adding linear polyacrylamide to 20  $\mu$ g/ml and filling the tube to the top with ethanol. The tubes are cooled, spun in a microfuge tube for three minutes, vortexed, and respun for one minute. The resulting pellets are rinsed with 70% ethanol and respun, taking care not to permit the pellets to dry to completion. Each 0.25 ml fraction is resuspended in 10  $\mu$ l of TE, and 1  $\mu$ l is run on a 1% agarose minigel. The first three fractions, and the last six which contain no material smaller than 1 kb are pooled.

#### 9e. Propagation of Plasmids

SupF plasmids are selected in nonsuppressing bacterial hosts containing a second plasmid, p3, which contains amber mutated ampicillin and tetracycline drug resistance elements. See Seed, Nucleic Acids Res., 11, 2427-2445 (1983). The p3 plasmid is derived from RP1, is 57 kb in length, and is a stably maintained, single copy episome. The ampicillin resistance of this plasmid reverts at a high rate so that  $amp^r$  plasmids usually cannot be used in p3-containing strains. Selection for tetracycline resistance alone is almost as good as selection for ampicillin-tetracycline resistance. However, spontaneous appearance of

chromosomal suppressor tRNA mutations presents an unavoidable background (frequency about  $10^{-9}$ ) in this system. Colonies arising from spontaneous suppressor mutations are usually larger than colonies arising from plasmid transformation. Suppressor plasmids are selected in Luria broth (LB) medium containing ampicillin at 12.5  $\mu\text{g/ml}$  and tetracycline at 7.5  $\mu\text{g/ml}$ . For scaled-up plasmid preparations, M9 Casamino acids medium containing glycerol (0.8%) is employed as a carbon source. The bacteria are grown to saturation.

Alternatively, pSV Sport (BRL, Gaithersburg, Maryland) may be employed to provide SV40 derived sequences for replication, transcription initiation and termination in COS 7 cells, as well as those sequences necessary for replication and ampicillin resistance in E. coli.

#### 9f. Isolation of Vector DNA/Plasmid

One liter of saturated bacterial cells are spun down in J6 bottles at 4.2k rpm for 25 minutes. The cells are resuspended in 40 ml 10 mM EDTA, pH 8. 80 ml 0.2M NaOH and 1% SDS are added, and the mixture is swirled until it is clear and viscous. 40 ml 5M KOAc, pH 4.7 (2.5M KOAc, 2.5M HOAc) is added, and the mixture is shaken semi-vigorously until the lumps are approximately 2-3 mm in size. The bottle is spun at 4.2k rpm for 5 minutes. The supernatant is poured through cheesecloth into a 250 ml bottle, which is then filled with isopropyl alcohol and centrifuged at 4.2k rpm for 5 minutes. The bottle is gently drained and rinsed with 70% ethanol, taking care not to fragment the pellet. After inverting the bottle and removing traces of ethanol, the mixture is resuspended in 3.5 ml Tris base/EDTA (20 mM/10 mM). 3.75 ml of resuspended pellet and 0.75 ml 10 mg/ml ethidium bromide are added to 4.5 g CsCl. VTi80 tubes are filled with solution, and centrifuged for at least 2.5 hours at 80k rpm. Bands are extracted by visible light with 1 ml syringe and 20 gauge or

lower needle. The top of the tube is cut off with scissors, and the needle is inserted upwards into the tube at an angle of about 30 degrees with respect to the tube at a position about 3 mm beneath the band, with the bevel of the needle up. After the band is removed, the contents of the tube are poured into bleach. The extracted band is deposited in a 13 ml Sarstedt tube, which is then filled to the top with n-butanol saturated with 1M NaCl extract. If the amount of DNA is large, the extraction procedure may be repeated. After aspirating the butanol into a trap containing 5M NaOH to destroy ethidium, an approximately equal volume of 1M ammonium acetate and approximately two volumes of 95% ethanol are added to the DNA, which is then spun at 10k rpm for 5 minutes. The pellet is rinsed carefully with 70% ethanol, and dried with a swab or lyophilizer.

#### 9g. Preparation of Vector for Cloning

20 µg of vector is cut in a 200 µl reaction with 100 units of BstXI (New York Biolabs) at 50°C overnight in a well thermostated, circulating water bath. Potassium acetate solutions (5 and 20%) are prepared in SW55 tubes as described above. 100 µl of the digested vector is added to each tube and spun for three hours, 50k rpm at 22°C. Under 300 nm UV light, the desired band is observed to migrate 2/3 of the length of the tube. Forward trailing of the band indicates that the gradient is overloaded. The band is removed with a 1 ml syringe fitted with a 20 gauge needle. After adding linear polyacrylamide and precipitating the plasmid by adding three volumes of ethanol, the plasmid is resuspended in 50 µl of TE. Trial ligations are carried out with a constant amount of vector and increasing amounts of cDNA. Large scale ligation are carried out on the basis of these trial ligations. Usually the entire cDNA prep requires 1-2 µg of cut vector.

#### 9h. Buffers

Loading Buffer: .5M LiCl, 10 mM Tris pH 7.5, 1 mM EDTA .1% SDS.

Middle Wash Buffer: .15M LiCl, 10 mM Tris pH 7.5, 1 mM EDTA .1% SDS.

RT1 Buffer: .25M Tris pH 8.8 (8.2 at 42°), .25M KCl, 30 mM MgCl<sub>2</sub>.

RT2 Buffer: .1M Tris pH 7.5, 25 mM MgCl<sub>2</sub>, .5M KCl, .25 mg/ml BSA, 50 mM dithiothreitol (DTT).

10X Low Salt: 60 mM Tris pH 7.5, 60 mM MgCl<sub>2</sub>, 50 mM NaCl, 2.5 mg/ml BSA 70 mM DME

10X Ligation Additions: 1 mM ATP, 20 mM DTT, 1 mg/ml BSA 10 mM spermidine.

10X Kinasing Buffer: .5M Tris pH 7.5, 10 mM ATP, 20 mM DTT, 10 mM spermidine, 1 mg/ml BSA 100 mM MgCl<sub>2</sub>

#### 9i. Transfection of cDNA encoding Ligands in Cos 7 Cells

Cos 7 cells are split 1:5 into 100 mm plates in Dulbecco's modified Eagles medium (DME)/10% fetal calf serum (FCS), and allowed to grow overnight. 3 ml Tris/DME (0.039M Tris, pH 7.4 in DME) containing 400 µg/ml DEAE-dextran (Sigma, D-9885) is prepared for each 100 mm plate of Cos 7 cells to be transfected. 10 µg of plasmid DNA preparation per plate is added. The medium is removed from the Cos-7 cells and the DNA/DEAE-dextran mixture is added. The cells are incubated for 4.5 hours. The medium is removed from the cells, and replaced with 3 ml of DME containing 2% fetal calf serum (FCS) and 0.1 mM chloroquine. The cells are incubated for one hour. After removing the chloroquine and replacing with 1.5 ml 20% glycerol in PBS, the cells are allowed to stand at room temperature for one minute. 3 ml Tris/DME is added, and the mixture is aspirated and washed two times with Tris/DME. 10 ml DME/10% FCS is added and the mixture is incubated overnight. The transfected Cos 7 cells are split 1:2 into fresh 100 mm plates with (DME)/10% FCS and allowed to grow.

## 9j. Panning Procedure for Cos 7 cells Expressing Ligand

### 1) Antibody-coated plates:

5 Bacteriological 100 mm plates are coated for 1.5 hours with rabbit anti-human placental alkaline phosphatase (Dako, California) diluted 1:500 in 10 ml of 50 mM Tris.HCl, pH 9.5. The plates are washed three times with 0.15M NaCl, and incubated with 3 mg BSA/ml PBS overnight. The blocking solution is  
10 aspirated, and the plates are utilized immediately or frozen for later use.

### 2) Panning cells:

5 The medium from transfected Cos 7 cells is aspirated, and 3 ml PBS/0.5 mM EDTA/0.02% sodium azide is added. The plates are incubated at 37°C for thirty minutes in order to detach the cells. The cells are triturated vigorously with a pasteur pipet and collected in a 15 ml centrifuge tube. The plate is washed with a further 2 ml PBS/EDTA/azide solution, which is then added to the centrifuge tube. After centrifuging at 200 xg for five minutes, the cells are resuspended in 3 ml of APTaq-flk-1 (F-1AP21-4) or flk-2 (F-2AP26-0) supernatant from transfected  
25 NIH/3T3 cells (see Example 7.), and incubated for 1.5 hours on ice. The cells are centrifuged again at 200 xg for five minutes. The supernatant is aspirated, and the cells are resuspended in 3 ml PBS/EDTA/azide solution. The cell suspension is layered carefully on 3 ml PBS/EDTA/azide/2% Ficoll, and centrifuged at 200 xg for four minutes. The supernatant is aspirated, and the  
30 cells are resuspended in 0.5 ml PBS/EDTA/azide solution. The cells are added to the antibody-coated plates containing 4 ml PBS/EDTA/azide/5% FBS, and allowed to stand at room temperature one to three hours. Non-adhering cells are removed by washing gently two or three times with 3 ml PBS/5% FBS.



### 3) Hirt Supernatant:

0.4 ml 0.6% SDS and 10 mM EDTA are added to the panned plates, which are allowed to stand 20 minutes. The viscous mixture is added by means of a pipet into a microfuge tube. 0.1 ml 5M NaCl is added to the tube, mixed, and chilled on ice for at least five hours. The tube is spun for four minutes, and the supernatant is removed carefully. The contents of the tube are extracted with phenol once, or, if the first interface is not clean, twice. Ten micrograms of linear polyacrylamide (or other carrier) is added, and the tube is filled to the top with ethanol. The resulting precipitate is resuspended in 0.1 ml water or TE. After adding 3 volumes of EtOH/NaOAc, the cells are reprecipitated and resuspended in 0.1 ml water or TE. The cDNA obtained is transfected into any suitable E. coli host by electroporation. Suitable hosts are described in various catalogs, and include MC1061/p3 or Electromax DH10B Cells of BRL Gibco. The cDNA is extracted by conventional methods.

The above panning procedure is repeated until a pure E. coli clone bearing the cDNA as a unique plasmid recombinant capable of transfecting mammalian cells and yielding a positive panning assay is isolated. Normally, three repetitions are sufficient.

### 9k. Expression cloning of flk1 or flk2 ligand by establishment of an autocrine loop

Cells expressing flk1/fms or flk2/fms (Example 10) are transfected with 20-30 µg of a cDNA library from either flk1 ligand or flk2 ligand expressing stromal cells, respectively. The cDNA library is prepared as described above (a-h). The cells are co-transfected with 1 µg pLTR neo cDNA. Following transfection the cells are passaged 1:2 and cultured in 800 µg/ml of G418 in Dulbecco's medium (DME) supplemented with 10% CS. Approximately 12 days later the colonies of cells are passaged

and plated onto dishes coated with poly -D- lysine (1 mg/ml) and human fibronectin (15 µg/ml). The culture medium is defined serum-free medium which is a mixture (3:1) of DME and Ham's F12 medium. The medium supplements are 8 mM NaHCO<sub>3</sub>, 15 mM HEPES pH 7.4, 3 mM histidine, 4 µM MnCl<sub>2</sub>, 10 uM ethanolamine, 0.1 µM selenous acid, 2 µM hydrocortisone, 5 µg/ml transferrin, 500 µg/ml bovine serum albumin/linoleic acid complex, and 20 µg/ml insulin (Ref. Zhan, X, et al. Oncogene 1: 369-376,1987). The cultures are refed the next day and every 3 days until the only cells capable of growing under the defined medium condition remain. The remaining colonies of cells are expanded and tested for the presence of the ligand by assaying for binding of APTag - flk1 or APTag - flk2 to the cells (as described in Example 8). The DNA would be rescued from cells demonstrating the presence of the flk1 or flk2 ligand and the sequence.

#### Example 10. Expression of Ligand cDNA

The cDNA is sequenced, and expressed in a suitable host cell, such as a mammalian cell, preferably COS, CHO or NIH/3T3 cells. The presence of the ligand is confirmed by demonstrating binding of the ligand to APTag-flk2 fusion protein (see above).

#### Example 11. Chemical Cross Linking of Receptor and Ligand

Cross linking experiments are performed on intact cells using a modification of the procedure described by Blume-Jensen et al et al., EMBO J., 10, 4121-4128 (1991). Cells are cultured in 100mm tissue culture plates to subconfluence and washed once with PBS-0.1% BSA.

To examine chemical cross linking of soluble receptor to membrane-bound ligand, stromal cells from the 2018 stromal cell line are incubated with conditioned media (CM) from transfected

3T3 cells expressing the soluble receptor Flk2-APtag. Cross linking studies of soluble ligand to membrane bound receptor are performed by incubating conditioned media from 2018 cells with transfected 3T3 cells expressing a Flk2-fms fusion construct.

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Binding is carried out for 2 hours either at room temperature with CM containing 0.02% sodium azide to prevent receptor internalization or at 4°C with CM (and buffers) supplemented with sodium vanadate to prevent receptor dephosphorylation. Cells are washed twice with PBS-0.1% BSA and four times with PBS.

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Cross linking is performed in PBS containing 250 mM disuccinimidyl suberate (DSS; Pierce) for 30 minutes at room temperature. The reaction is quenched with Tris-HCL pH7.4 to a final concentration of 50 mM.

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Cells are solubilized in solubilization buffer: 0.5% Triton - X100, 0.5% deoxycholic acid, 20 mM Tris pH 7.4, 150 mM NaCl, 10mM EDTA, 1mM PMFS, 50 mg/ml aprotinin, 2 mg/ml bestatin, 2 mg/ml pepstatin and 10mg/ml leupeptin. Lysed cells are immediately transferred to 1.5 ml Nalgene tubes and solubilized by rolling end to end for 45 minutes at 4°C. Lysates are then centrifuged in a microfuge at 14,000g for 10 minutes. Solubilized cross linked receptor complexes are then retrieved from lysates by incubating supernatants with 10% (v/v) wheat germ lectin-Sepharose 6MB beads (Pharmacia) at 4°C for 2 hours or overnight.

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Beads are washed once with Tris-buffered saline (TBS) and resuspended in 2X SDS-polyacrylamide nonreducing sample buffer. Bound complexes are eluted from the beads by heating at 95°C for 5 minutes. Samples are analyzed on 4-12% gradient gels (NOVEX) under nonreducing and reducing conditions (0.35 M 2-mercaptoethanol) and then transferred to PVDF membranes for 2

hours using a Novex blotting apparatus. Blots are blocked in TBS-3% BSA for 1 hour at room temperature followed by incubation with appropriate antibody.

5 Cross linked Flk2-Aptag and Flk2-fms receptors are detected using rabbit polyclonal antibodies raised against human alkaline phosphatase and fms protein, respectively. The remainder of the procedure is carried out according to the instructions provided in the ABC Kit (Pierce). The kit is based on the use of a  
10 biotinylated secondary antibody and avidin-biotinylated horseradish peroxidase complex for detection.

Example 12. Expression and purification of Flag-Flk-2.

1. Design of the Flag-Flk2 expression plasmids.

20 A synthetic DNA fragment (Fragment 1) is synthesized using complementary oligonucleotides BP1 and BP2 (see below and SEQ. ID. NOS. 7 and 8). The fragment encoded the following features in the 5' to 3' order: Sal I restriction site, 22 base pair (bp) 5' untranslated region containing an eukaryotic ribosome binding site, an ATG initiation codon, preprotrypsinogen signal sequence, coding region for the FLAG peptide (DYKDDDDKI) and Bgl II  
25 restriction site.

30 A cDNA fragment (Fragment 2) encoding Asn 27 to Ser 544 of murine Flk2 is obtained by polymerase chain reaction (PCR) using primers designed to introduce an in frame Bgl II site at the 5' end (oligonucleotide BP5, see below and SEQ. ID. NO. 9) and a termination codon followed by a Not I site at the 3' end (oligonucleotide BP10, see below and SEQ. ID. NO. 10). The template for the PCR reaction is full length Flk2 cDNA (Matthews et al., Cell 65:1143 (1991)). Fragment 2 is extensively digested  
35 with Bgl II and Not I restriction enzymes prior to ligation.

To assemble the complete Flag-Flk2 gene, Fragments 1 and 2 are ligated in a tripartate ligation into Sal I and Not I digested plasmid pSPORT (Gibco/BRL, Grand Island, NY) to give the plasmid pFlag-Flk2.

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Preferably, the Flag-Flk2 protein is attached at either end to the Fc portion of an immunoglobulin (Ig). The Ig is preferably attached to the Flk2 portion of the Flag-Flk2 protein. To assemble the construct pFlag-FLK2-Ig, the sequences coding for the CH<sup>1</sup> domain of human immunoglobulin G (IgG<sup>1</sup>) are placed downstream of the Flk2 coding region in the plasmid pFlag-Flk2 as per the method described by Zettlemessl et al., DNA and Cell Biology 9: 347-352 (1990).

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The sequences of oligonucleotides used to construct the Flag-Flk2 gene are given below:

Oligonucleotide BP1:

5'-AATTCGTCGACTTTCTGTCACCATGAGTGCACCTTCTGATCCTAGCCCTTGTG  
GGAGCTGCTGTTGCTGACTACAAAGATGATGATGACAAGATCTA-3'

Oligonucleotide BP2:

5'-AGCTTAGATCTTGTTCATCATCTTTGTAGTCAGCAACAGCAGCTCCCACA  
AGGGCTAGGATCAGAAGTGCACCTCATGGTGACAGAAAGTCGACG-3'

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Oligonucleotide BP5:

5'-TGAGAAGATCTCAAACCAAGACCTGCCTGT-3'

Oligonucleotide BP10:

5'-CCAATGGCGGCCGCTCAGGAGATGTTGTCTTGA-3'

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## 2. Expression of the Flag-Flk2 construct.

For transient expression of the Flag-Flk2 construct, the Sal I to Not I fragment from pFlag-Flk2 is subcloned into the

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plasmid pSVSPORT (Gibco/BRL) to give the plasmid pSVFlag-Flk2. For expression of the Flag-Flk2 protein pSVFlag-Flk2 is transfected into COS monkey cells using the DEAE-dextran method.

5 For stable expression in eukaryotic cells, the Sal I-Not I fragment of pFlag-Flk2 is cloned into the EcoRV and Not I sites of the plasmid pcDNA I/Neo (Invitrogen Co., San Diego, CA). The Sal I 3' recessed terminus of pFlag-Flk2 is filled with the Klenow fragment of DNA polymerase I and a mixture of  
10 deoxyribonucleotides to make the site compatible with the EcoRV site of the vector. The resulting construct is introduced into cultured mamalian cells using either the Lipofectin (Gibco/BRL) or the calcium phosphate methods.

For expression in insect cells, the SalI to Hind III (from pSPORT polylinker) fragment of pFlag-Flk2 is subcloned into the BamHI-Hind III sites of the baculovirus transfer vector pBlueBac III (Invitrogen). The vector Bam HI site and the insert Sal I site are blunted with Klenow (see above). Production of the recombinant virus and infection of the Sf9 insect cells is performed as per manufacturers directions (Invitrogen).

20 Expression of the Flag-Flk2 protein is detected by Western blotting of SDS-PAGE separated conditioned media (mamalian cells) or cell lysates (insect cells) with the anti-Flag monoclonal antibody (mAb) M1 (International Biotechnology, Inc. [IBI], New Haven, CT).

3. Affinity purification of the Flag-Flk2 protein from  
30 conditioned media or insect cell lysates is performed using immobilized mAb M1 (IBI) as per manufacturers specifications.

3.1 Affinity purification of the Flag-Flk2-Ig<sup>1</sup> protein from conditioned media is performed using immobilized Protein A  
35 (Pharmacia LKB, Piscataway, NJ) as per the manufacturers

instructions.

## II. Use of the Flag-Flk2 protein to search for the Flk2 ligand.

1. Binding and cross-linking studies to detect membrane-bound ligand:

### A. Binding studies.

Murine stromal lines ( eg. 2018 cells ATCC CRL 10907 (see below), see example 1, *supra*) considered to be candidates for expression of the Flk2 ligand were deposited at the American Type Culture Collection, ATCC CRL 10907 (see below) and cultured in Dulbecco's modified Eagles medium (DMEM; Gibco/BRL) supplemented with 10% fetal calf serum. The cells are grown to confluency in 10 cm plates and washed once with PBS. Conditioned media containing Flag-Flk2 is incubated with the cells at 4°C for 2 hrs. The cell monolayers are rinsed extensively to remove the non-bound protein, solubilized and centrifuged to remove insoluble cellular material. Glycoproteins in the lysates are partially purified with wheat germ agglutinin-Sepharose (Pharmacia LKB, Piscataway, NJ), boiled in an SDS sample buffer, separated on SDS-PAGE gels and transferred to nitrocellulose membranes. The membranes are probed with the M1 antibody to detect the presence of cell-associated Flag-Flk2 protein.

B. In a cross-linking study, the above protocol is followed except that prior to solubilization the monolayer are treated with the crosslinker disuccinimidyl suberate (DSS; Pierce, Rockford, IL). The presence of a putative ligand is detected by an upward shift in the apparent molecular weight of the Flag-Flk2 band on Western blots.

C. Purified Flag-Flk2 protein labelled with Na<sup>125</sup>I via the Chloramine T method is used to asses the ability of the soluble

extracellular domain of the Flk2 receptor to bind transmembrane form of the Flk2 ligand in cultured stromal lines. The labelled protein is added to monolayers of stromal cells on ice for 2 hr in the presence or absence of excess unlabelled protein. Specific binding is calculated by subtracting counts bound in the presence of excess unlabelled protein from the total counts bound.

2. Use of the Flag-Flk2 protein to search for secreted form of the ligand.

A. The Flag-Flk2 protein is used in attempts to identify the Flk2 ligand in conditioned media from stromal cell cultures via modification of the direct N-terminal sequencing method of Pan et al., Bioch. Biophys. Res. Comm. 166:201 (1990). Briefly, the Flag-Flk2 protein N-terminally sequenced by automatic Edman degradation chemistry on an ABI 477A sequencer with on line PTH amino acid analysis. Approximately 15 amino acids are determined. The protein is then immobilized on Nugal PAF silica beads via free NH<sub>4</sub><sup>+</sup> groups. The immobilized Flag-Flk2 is incubated with conditioned media from putative ligand-producing cells for 30 min at 4°C and washed free off non-bound proteins with phosphate buffered saline adjusted to 2M NaCl. The resulting protein complex is resequenced. For each sequencing cycle, any amino acid not expected at this position in the FLAG-Flk2 protein is considered as possibly originating from a protein complexed to the Flk2 receptor.

B. For conventional affinity chromatography, the Flag-Flk2 protein is immobilized on a stable support such as Sepharose. 35S-methionine labelled-conditioned media from stromal cell lines are passed over the affinity matrix and bound material is analyzed by SDS-PAGE gel electrophoresis and autoradiography.

3. Use of the Flag-Flk2 protein in expression cloning experiments.



5 A method of expression cloning of integral membrane proteins  
in COS cells has been described (Aruffo and Seed, Proc. Natl.  
Acad. Sci. 84:8573 (1987)). A cDNA library is prepared from an  
appropriate stromal cell line such as 2018 and is transfected  
into COS cells. Cells transiently expressing the Flk2 ligand are  
affinity adsorbed onto plastic plates coated with the Flag-Flk2  
protein. The cells are lysed, the plasmid DNA is recovered and  
amplified in a bacterial host. The cycle of transfection into COS  
cells is repeated until a single cDNA clone encoding the ligand  
molecule is isolated.

10 In a modification of the above technique, pools of  
transfected COS cells are screened for binding of 125I-Flag-Flk2.  
Positive cells pools are selected and plasmid DNA is recovered  
and amplified in E. coli. The resulting DNA preparation is used  
in subsequent rounds of transfection and transient expression  
until all cells are positive for binding of 125I-Flag-Flk2. The  
cDNA in the final plasmid preparation is then sequenced to  
determine the sequence of the putative Flk-2 ligand.

#### SUPPLEMENTAL ENABLEMENT

25 The invention as claimed is enabled in accordance with the  
above specification and readily available references and starting  
materials. Nevertheless, Applicants have deposited with the  
American Type Culture Collection, Rockville, Md., USA (ATCC) the  
cell lines listed below:

30 2018, ATCC accession no. CRL 10907, deposited  
October 30, 1991.

Fsp 62891, ATCC accession no. CRL 10935, deposited  
November 21, 1991.

35 F.thy 62891, ATCC accession no. CRL 10936,

deposited November 21, 1991.

FL 62891, ATCC accession no. CRL 11005, deposited April 2, 1992.

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These deposits were made under the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure and the regulations thereunder (Budapest Treaty). This assures maintenance of a viable culture for 30 years from date of deposit. The organisms will be made available by ATCC under the terms of the Budapest Treaty, and subject to an agreement between Applicants and ATCC which assures unrestricted availability upon issuance of the pertinent U.S. patent. Availability of the deposited strains is not to be construed as a license to practice the invention in contravention of the rights granted under the authority of any government in accordance with its patent laws.

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